INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed Andrew Gregg

Dated 7 November 2003

Patents Form 1/77

Patents Act 1977
(Rule 16)

THE PATENT OFFICE
PATENTS • DESIGNS • COPYRIGHT • TRADE MARKS
Patent Office
14 NOV 2002
LONDON

15NOV02 E763615-1 D00192
P01/7700 0.00-0226598.1

The Patent Office

Cardiff Road
Newport
South Wales
NP10 8QQ

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

1. Your reference — P86939 PEJ

2. Patent application number *(The Patent Office will fill in this part)* — 0226598.1 — 14 NOV 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Isis Innovation Limited
Ewert House, Ewert Place
Summertown
Oxford OX2 7SG

Patents ADP number *(if you know it)* 8408981001

If the applicant is a corporate body, give the country/state of its incorporation — British body corporate

4. Title of the invention — CHEMICAL INHIBITORS

5. Name of your agent *(if you have one)* — J.A. KEMP & CO.

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

14 South Square
Gray's Inn
London
WC1R 5JJ

Patents ADP number *(if you know it)* 26001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number	Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

Yes

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	22
Claim *(s)*	5 DML
Abstract	
Drawing *(s)*	

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature J.A. KEMP & CO. Date 14 11 2002

12. Name and daytime telephone number of person to contact in the United Kingdom

PGA Ellis-Jones
020 7405 3292

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 08459 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

CHEMICAL INHIBITORS

Field of Invention

The present invention relates to chemical inhibitors of FIH and their use in the treatment of ischaemia.

Background of the Invention

In cells of many organisms exposure to an environment in which oxygen is depleted relative to optimal levels induces a hypoxic response. In these hypoxic cells, activation of a transcriptional cascade involving hypoxia inducible factor (HIF) directs a series of adaptive responses that enhance oxygen delivery or limit oxygen demand. Activation of HIF in cancer and ischaemic hypoxic vascular diseases has revealed its important role in human pathology and demonstrated that manipulation of HIF activity has important therapeutic potential.

The HIF transcriptional complex comprises an αβ heterodimer, HIF-β being a constitutive nuclear protein that dimerises with oxygen regulated HIF-α subunits (Semenza, G. L. (2000) *Genes Dev.* **14**, 19831991). The activity of HIF-α, is suppressed by oxygen-dependent modification catalysed by a series of $Fe^{(II)}$ and 2OG dependent dioxygenases that hydroxylate specific HIF-α residues. In the presence of oxygen in human HIF-1α, 4-hydroxylation of Pro402 or Pro564 by a set of HIF prolyl hydroxylase isozymes (PHD1-3) (Epstein et al. (2001) *Cell* **107**, 4354; Bruick, R. K., and McKnight, S. L. (2001) *Science* **294**, 13371340) mediates its recognition by the von Hippel-Lindau (VHL) ubiquitin ligase complex and consequent targeting for proteasomal destruction (Ivan et al, (2001) *Science* **292**, 464468; Jaakkola et al (2001) *Science* **292**, 468472). In a complementary mechanism FIH catalyses β-hydroxylation of HIF-1α Asn803 (Lando et al, (2002) *Science* **295**, 858861) blocking interaction with the transcriptional co-activator p300 (Dames et al., (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 52715276; Freedman et al, (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 53675372). In hypoxia, limitation of enzymatic activity

allows HIF-α to escape destruction and become transcriptionally active.

Inhibition of HIF hydroxylases strongly activates the HIF transcriptional cascade even in the presence of oxygen (Epstein et al.(2001) *Cell* **107**, 4354). Thus, inhibition of the HIF hydroxylases results in a pro-angiogenetic response that may be used in the treatment of cardiovascular diseases/ ischaemic hypoxic vascular diseases including myocardial infarction and anaemia. A problem with this approach is that the human cells contain other enzymes belonging to the same family as the HIF hydroxylases, i.e. utilising dioxygen (a cosubstrate) 2-oxoglutarate (2OG) (a cosubstrate) and Fe(II) (a cofactor). Such enzymes are exemplified by phytanoyl coenzyme A hydroxylase, procollagen prolyl-4-hydroxylase, procollagen prolyl-3-hydroxylase, gamma-butyrobetaine hydroxylase, Alk B (a DNA repair enzyme) and others including predicted 2OG oxygenases identified on the basis of sequence analyses including a sub-family related to FIH (Hewitson et al., J BIOL CHEM 277 (29): 26351-26355, 2002). It is generally agreed that it is desirable that enzyme inhibitors used as pharmaceuticals are selective for their intended target or the targets involved in producing the desired effect. A lack of selectivity can lead to toxic side effects that render particular compounds unsuitable for use in human or animal therapy. One approach to identifying compounds that are selective for the intended target is to undertake structural, mechanistic and other analyses on the intended agents and to use the information gained to aid in the preparation of selective compounds, or more selective compounds (relative to those previously known), for use as pharmaceuticals for use in humans or animals. Here we describe structural and other studies on the HIF hydroxylases that enable the design of selective inhibitors of FIH and related enzymes.

The inventors have now identified the site of hydroxylation of asparagine-803 of HIF-1α by FIH. In addition, they have obtained the crystal structure for FIH including identification of the binding site and residues involved in the interaction of FIH with HIF. This invention forms the subject of our Application No. 0224102.4 filed on 16th October 2002 (N.86855).

Summary of the Invention

We have determined the structure of various families of inhibitors of FIH.

Detailed Description of the Invention

The present inventors have identified the position of asparagine 803 that is hydroxylated by FIH and have devised chemical entities which can bind and in particular which can inhibit FIH. A number of different types of inhibitors can be identified as discussed in more detail below.

Inhibitors exploiting metal binding:

The structural work defines the presence of Fe(II) at the active site of FIH and implication related HIF hydroxylases. The iron is bound in an almost octahedral manner by the side chains of His-199, Asp-201 and His-279, the 2-oxo and 1-carboxylate groups of 2OG. In the enzyme-substrate complexes there is a vacant position opposite His-279 revealing that the enzyme is primed for dioxygen binding. Accommodation of a ligand opposite His-279 may require disruption of the hydrogen bond between Asp-201 and CAD Asn-803 (the iron and Asn-803 β-carbon are only ~4.9 Å apart). Subsequent decarboxylation of 2OG presumably yields an iron-oxo species [$Fe^{(IV)}$=O <--> $Fe^{(III)}$-O•] that effects oxidation at the carbon of Asn-803 in the C-terminal transactivation domain (CAD) of HIF.

Compounds that contain functional groups that bind to iron, and in particular the iron in FIH, are useful as inhibitors of FIH.

$Zn^{(II)}$ binds to FIH in an identical manner to $Fe^{(II)}$ (structure 3), consistent with the metal-mediated hypoxic effect being due to displacement of $Fe^{(II)}$ from the active site of HIF hydroxylases. Since neither Zn(II) nor other metal inhibitors of FIH can replace Fe(II) as a cofactor in catalysis, compounds that preferentially promote the binding of a metal other than iron [such as Zn(II)] at the active site of FIH act as inhibitors.

A further class of inhibitor are non-metallic inhibitors that operate via competing with Fe(II) for binding at the active site. Such inhibitors may bind to any

or all of the triad of residues (His-199, Asp-201, His-279), that bind the Fe(II) at the active site of catalytically active FIH.

Compounds that contain functional groups that bind to iron include thiols, alcohols, phenols, including flavonoids such as quercetin and derivatives thereof, carboxylates, hydroxamates, imidazoles and other heterocycles e.g. nitrogen-containing heterocycles.

Inhibitors exploiting the 2OG binding sites

The FIH:CAD structures with NOG (N-oxaloylglycine) reveal that like 2OG it is ligated to iron in a bidentate manner and imply it is an inhibitor due to decreased susceptibility to attack by an iron bound (su)peroxide intermediate or by hindering binding of dioxygen to the metal.

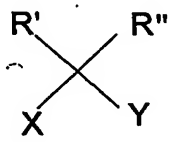
The structural studies on FIH reveal the binding interactions for the 2OG and NOG. The 5-carboxylate of 2OG (and the equivalent carboxylate of NOG) forms hydrogen bonds with the side-chains of Lys-214, Thr-196 and Tyr-145; such interactions are unprecedented in other structures of 2OG oxygenases. FIH is further unusual in that Lys-214 is on the fourth DSBH (double stranded beta-helix) β-strand whereas previously assigned basic 2OG-5-carboxylate binding residues are at the beginning of the eighth DSBH strand.

The structural studies reveal the FIH residues that form the pocket into which 2OG and NOG bind. In addition to the aforementioned these include the side-chains residues of Ile-281, Leu-186, Leu-188, Phe-207, Thr-196. Knowledge of these interactions enables the design of improved (as measured by binding parameters) and selective inhibitors. Thus, for example an inhibitor binding in the 2OG binding pocket may form hydrophobic interactions with any or all of the side chains of Ile-281, Leu-186 Leu-188, Phe-207, Thr-196. Further it may form electrostatic or hydrogen bonding interactions with the residues involved in binding the 5-carboxylate of 2OG (Lys214, Thr196 and Tyr145).

Selective inhibition of FIH via inhibitors interacting with the 2OG binding residues is exemplified as follows: kinetic analyses of a series of inhibitors based

upon *N*-oxaloyl amino acids revealed the *R*-enantiomer (IC_{50} 0.4 mM) of *N*-oxaloylalanine was significantly more potent than the *S*-enantiomer (IC_{50} 2.5 mM). Analysis of the 2OG binding pocket in FIH reveals that the binding of the S-enantiomer is hindered by interactions between its methyl group and the side chain of Thr-196 and, Ile-281 in the 2OG binding pocket. A reversed selectivity (i.e. the S-enantiomer was more potent) was observed both for procollagen prolyl-hydroxylase and the PHD isozymes, demonstrating it should be possible to develop selective inhibitors for individual types of HIF hydroxylase. Such inhibitors may or may not chelate to an active site metal.

Compounds include those of general formula

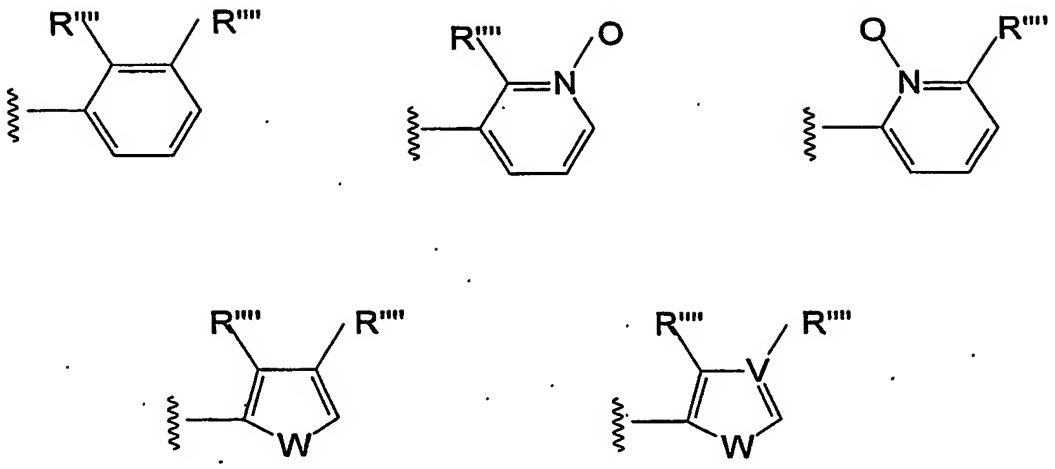


(I)

wherein each of R′ and R", which may be the same or different, is H, F or C_1 to C_3 alkyl or substituted alkyl, CH_2OH, CH_2CO_2H or $CONH_2$, X is COOH, SOOH, or CONHH or an ester thereof, or heterocyclic or other group which forms a favourable interaction with one or more of the side chains of Lys-214, Thr-196 and Tyr-145, i.e. those residues involved in binding the 5-carboxylate of 2OG as revealed in the crystallographic analyses,

Y is - $(CR'''R''')_nZ$, where Z is

- $NR'''COCOOH$, $-NR'''CSCOOH$, - $NR'''COCOSH$,
- $CHSR'''CONR'''R''''$, $-CHOR'''CONR'''OR'''$, - $CHSR'''CONR'''OR'''$ or
- $CHOR'''CONR'''NR'''OR'''$, wherein each R′′′, which may be the same or different, is H, alkyl, OH or O-alkyl, n is 0 to 3 and preferably 0, or



wherein R'''' is OH, OR''' or NHCOR''', and W is S, NH, or O.

Thus X is a group that forms favourable interactions with one or more of the side chains of interactions one or more of the side chains of Lys-214, Thr-196 and Tyr-145, i.e. those residues involved in binding the 5-carboxylate of 2OG. X may be functionalised as a pro-drug such that is delivered to the desired site of action or has desirable pharmokinetic properties. As indicated above, X can be an ester such a methyl or ethyl ester or amide derivative of carboxylic acid versions of X.

If n is 0, Y is typically CONHOH, $CONHNH_2$, NR'''COCOOH, NR'''CSCOOH or NR'''COCOSH. Y is preferentially of a size such that it can chelate to the active site metal whilst maintaining all or some of the favourable binding interactions found in the 2OG binding pocket as defined by crystallographic analyses. As with X, Y may be functionalised as a pro-drug.

When Y contains an aromatic ring as indicated above it can comprise other ring systems including aryl or functionalised aryl rings as well as heterocyclic and

functionalised heterocyclic rings. The above rings may be further functionalised to optimise binding at the FIH active site.

Inhibitors exploiting the peptide substrate binding site

There are two binding sites

The ES complex structures unexpectedly reveal two separate binding sites involving $CAD_{795-806}$ (i.e residues 795-806 of the C-terminal transactivation domain of HIF - 1 alpha) (Site 1) and $CAD_{813-822}$ of HIF - 1 alpha (Site 2) with contact surface areas of 1640 $Å^2$ and 1080 $Å^2$, respectively . CAD residues in these regions are conserved in all known HIF-1α and HIF-2α sequences. The electron density for site 1 was of good quality, with only the side-chain of Tyr-798 poorly defined, while that for site 2 was at a lower level and quality, probably reflecting weaker binding at this site. $CAD_{804-806}$ and presumably also $CAD_{807-811}$, for which density was not observed, do not form direct interactions with FIH. Kinetic analyses employed to investigate the relative importance of Sites 1 and 2, revealed that fragments containing site 1 only are hydroxylated by FIH but less efficiently than those containing both sites demonstrating that both are important in binding and that both may be exploited in inhibition studies.

At Site 1 $CAD_{795-803}$ are bound in a groove and adopt a largely extended conformation linked to FIH by ten hydrogen bonds. Asn-803 of CAD is strikingly buried at the active and directly adjacent to the $Fe^{(II)}$. CAD Asn-803 and Ala-804 form a tight turn, stabilised by a hydrogen bond between the backbone carbonyl of Val-802 and NH of Ala-804, which projects the side chain of Asn-803 towards the $Fe^{(II)}$. The side chain of CAD Asn-803 is orientated by three hydrogen bonds to enable hydroxylation at the *pro-S* position of the β-carbon consistent with the NMR assignments. The primary amide of CAD Asn-803 is sandwiched between FIH residue Tyr-102 and the $Fe^{(II)}$, and forms hydrogen bonds with the side chains of FIH residues Gln-239 and Arg-238 , residues located on the insert to the DSBH motif. Significantly, the substrate and $Fe^{(II)}$ binding sites are directly linked since the backbone nitrogen of CAD Asn-803 also forms a hydrogen bond (~3 Å) with the

carboxylate oxygen of Asp-201 that is not complexed to the iron. Six additional hydrogen bonds stabilise the binding of FIH to $CAD_{795-801}$.

In contrast with Site 1, Site 2 is located on the FIH surface and involves only two hydrogen bonds. $CAD_{816-823}$ of Site 2 form an α-helix, in exact agreement with the structure of this region in complex with CBP/p300 (Dames et al., (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 52715276; Freedman et al, (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 53675372). As in that complex, the highly conserved Leu818, Leu819 and Leu822 sit in a hydrophobic pocket on the surface of FIH and form the basis of the binding interaction and so it is not possible for these residues to bind simultaneously to CBP/p300 and FIH.

The extended loop conformation adopted by the CAD residues at Site 1, contrasts with the α-helical conformation adopted by the same residues when complexed with the 1st transcriptional adaptor zinc-binding domain (TAZ1) of CBP/p300(Dames et al.,(2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 52715276; Freedman et al, (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 53675372). The disordered structure observed for the CAD, and other HIF-α residues, when free in solution may thus reflect a requirement to adopt more than one conformation for complex formation with different proteins.

The changes in the conformation of CAD on binding are complemented by changes in FIH revealing an induced fit binding process; Trp-296 of FIH undergoes a 50° rotation about C_{beta}-C_{alpha} to accommodate CAD Val-802, while both Tyr-102 and Tyr-103 become more ordered. Further evidence of induced fit comes from the significant differences in resolution between the structures obtained with and without CAD fragments bound reflecting ordering of FIH that occurs on binding (structure 4, for comparison, represents FIH complexed with $Fe^{(II)}$ and 2OG alone). Interference in the conformational changes involved in the hypoxic response, in particular those involving the CAD region, e.g. by use of small molecules or by gene or protein therapy, may allow manipulation of the hypoxic response to enable pro or anti-angiogenetic responses.

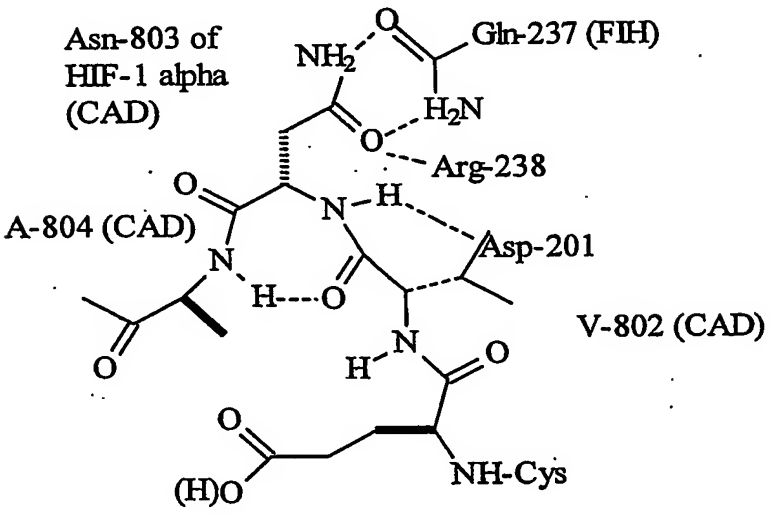
Thus, the structural studies define the (i) FIH residues involved in binding the CAD of HIF (ii) conformation of FIH when CAD is bound and (iii) conformation of

CAD when bound to FIH. These results are useful in the design of selective inhibitors of FIH and related enzymes. Features of the FIH binding Sites may be used to mediate tighter binding of inhibitors to FIH or to obtain inhibitors that do not bind tightly to FIH, i.e. avoid inhibition of FIH.

Inhibitors binding at or close to the Site 1 may exploit electrostatic, hydrogen binding and/or hydrophobic interactions with Tyr-102, Asp-104, Lys-106, Asp-201, Glu-202, Gln-147, Gln-239, residues 299-303, His-313, Ala-317, Ile-318, Asn-321, Lys-324, Arg-238, Trp-296, Asn-321- Lys-324. Inhibitors binding at Site 1 may mimic or partially mimic the turn conformation adopted by CAD when bound at Site 1.

Inhibitors binding at or close to Site 2 may exploit electrostatic, hydrogen binding and/or hydrophobic interactions with residues Thr-149, Leu-150, Asn- 151, Asp-152 and residues Val-159, Phe-162, Leu-163, Trp-167, Gln-181, Leu-182, Thr-183, Ser- 184, Asn- 185. Inhibitors binding at Site 2 may mimic or partially mimic the helical conformation adopted by CAD when bound at Site 2.

It is recognised that inhibitors need not bind to both Sites 1 and 2, although that they may, and that Site 1 is preferred over Site 2.



Residues 801 – 805 of CAD that bind at Site 1, and in particular residues 802-805 form a turn conformation in which the distance of the backbone C=O of 802 to the backbone NH of 804 is ca. 2.8 Å. Including the H-bond formed between the NH of Ala-804 and the carbonyl O of Val-802 of the HIF-1alpha CAD, the turn contains 7 atoms in a pseudo-ring.

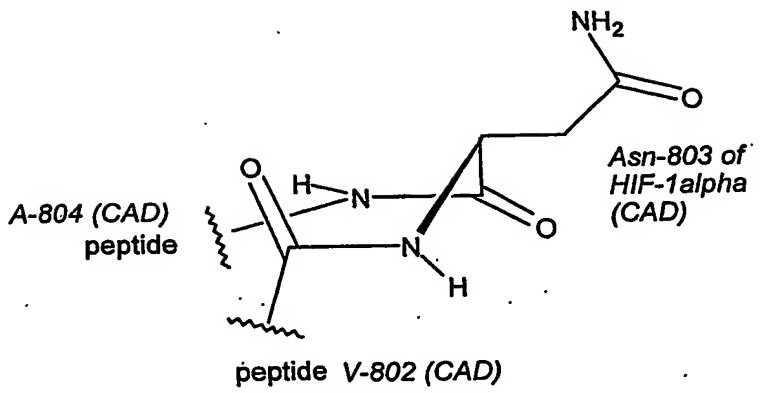


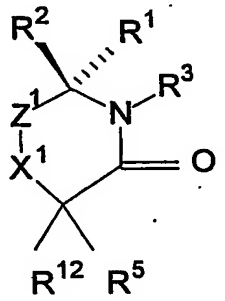
Figure indicating conformation of the turn formed by resic
802-804 of HIF-CAD at the active site of FIH.

Turns are especially amenable to mimicry by analogues useful for enzyme inhibition or receptor binding.

The medicinal chemistry literature is replete with examples of such turn mimics. Most can be modified via known methods to bind to specific targets. They can be modified either by knowledge of the target structure and/or by iterative methods. Further, turn mimics have been prepared in a combinatorial manner via solid phase or parallel synthesis. Examples of turn mimics and their modifications can be found in the following reviews: Hanessian et al, TETRAHEDRON 53: 12789-12854 SEP 22 1997; Gillespie et al, BIOPOLYMERS 43: 191-217 1997; Burgess K et al, ACCOUNTS CHEM RES 34: 826-835 2001. Recent examples of primary reports on turns include the following (and references therein) Maier et al, EUR J ORG CHEM: 2686-2689, 2002; Reid et al J AM CHEM SOC 124: 5673-5683, 2002; Mahadevan et al, J BIOMOL STRUCT DYN 19: 775-788 2002; Fguchi et al, J MED CHEM 45: 1395-1398 2002; De Borggraeve et al, TETRAHEDRON LETTERS 42: 5693-5695 2001; Kohn et al, TETRAHEDRON LETT 42: 4453-4457 2001; Eguchi et al, TETRAHEDRON LETT 42: 1237-1239 2001; Manzoni et al, TETRAHEDRON 57: 249-255 2001; Jiang et al, HELV CHIM ACTA 83: 3097-

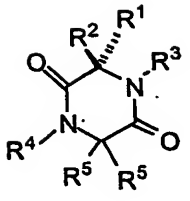
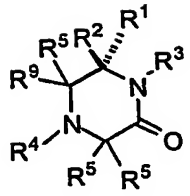
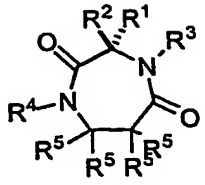
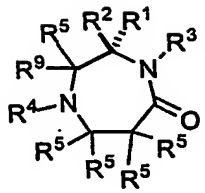
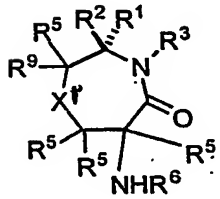
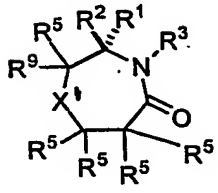
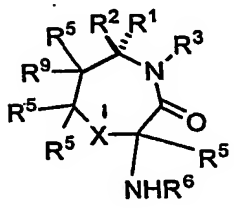
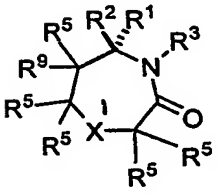
3112 2000; Derrer et al, J CHEM SOC PERK T 1: 2957-2967 2000; Belvisi et al EUR J ORG CHEM: 2563-2569 2000; Claridge et al BIOORG MED CHEM LETT 6: 485-490 1996.

These include compounds of the general formula:



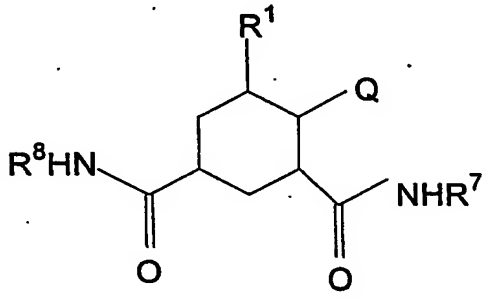
II

wherein R^1 is such that it can form an electrostatic or H-bonding interaction with Gln-237 and or Arg-238, preferably $CR^8R^9CONH_2$ or an analogue thereof where R^8 is hydrogen or a peptide or peptide mimetic (such as those composed of β-amino acids or peptide isoteres), and R^9 is hydrogen, optionally functionalised alkyl, optionally functionalised aryl, heteroaryl or any combination thereof such as CH_2CONH_2, R^2 is hydrogen or a group that will interact favourably with Tyr-102 of FIH, R^3 is H or a group which can form a H-bond with Asp-201, Z^1 is $\rangle C{=}O$ or $\rangle CR^5R^9$ where R^5 is hydrogen, optionally functionalised alkyl, aryl, or heteroaryl or any combination thereof, R^{12} is as defined for R^5 or is NHR^6 where R^6 is COR^5 or $SO_2R^5$and X^1 is NR^4, $NR^4C(R^5)_2$, $C(R^5)_2NR^4$, or O or NH where R^4 is COR^5 or SO_2R^5. In this and in the other formulae each R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^8, R^9, R^{10}, R^{11} and R^{12} can be the same or different. In particular, these compounds may have one of the formulae

wherein the radicals are as defined above, and R^7 and R^8 are independently peptides or peptides mimetics or part peptide mimetics, such as those containing or consisting of beta-amino acid residues, urethane, sulphonamide or phosphonamide links.

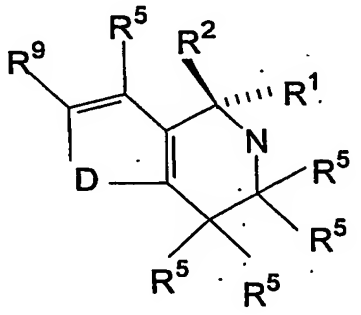
Other compounds which can be used are those possessing the formula



III

where Q represents H or OH and R^7 and R^8 are as defined above.

Further compounds which can be used possess the formula



IV

wherein R^1, R^2, R^5 and R^9 are as defined above and D is S, O, NH or CHR^9=CHR^9. Thus the ring attached to the six-membered ring is either a five-membered heterocyclic ring or an aryl ring.

In these formulae R^8 and R^9 can be optimised to bind in the channel linking the 2OG and peptide substrate binding sites and to the 2OG binding site itself.

Cyclic peptides acting as mimics of the turn adopted by CAD in site 1. The cyclo may be formed via peptide links, disulphide bonds or C-C bonds.

Inhibitors employing a combination of binding sites

It is well known that enzyme inhibitors competing for binding at more than one substrate or cosubstrate binding site, sometimes termed bisubstrate inhibitors, can be useful. Examples can be found in Wang et al, BIOCHEMISTRY-US: 15676-15683 2001; Lerner et al, ANGEW CHEM INT EDIT 40, 4040-4041, 2001. In the case of FIH and other 2OG oxygenases bisusbtrate inhibitors may be useful since features of 2OG binding may be present in more than one enzyme whereas the CAD substrate is unique. Thus, inhibitors that bind to both binding sites may show improved selectivity over those that bind to the 2OG binding site only. The structural analyses enable the identification of such bisusbtrate inhibitors. The 2OG and CAD binding sites are linked to each other via a 'channel' extending from the 2-oxo group of 2OG (or NOG) to the beta-carbon Asn-803 in the FIH.Fe.2OG/NOG.HIF(CAD) complexes. In the structures this 'channel' either appears empty but may be occupied by water molecules. The distance from the C of the 2-oxo group of 2OG to the beta-C of Asn-803 is ca. 6 Å. The distance from the 3-C of 2OG to the beta-C of Asn-803 is ca. 6.5 Å. The information from the structural analyses enables the identification of bisubstrate inhibitors, including the following:

These are compounds of formulae (II) to (IV) as defined above except that they are modified such that they can also bind into the 2OG binding pocket as defined by the crystallographic information. Thus, either R^2 or R^1 is modified such that they can bind into the 2OG binding pocket. The modification takes the form such that the general formula of R^1 or R^2 is A-X where X is as defined above and A

links X to (II). A is of appropriate length such that X can bind to formula 1 the residues of the 5-carboxylate of 2OG as discussed above under the heading Inhibitors Exploiting the 2OG binding sites.

More generally bi-substrate inhibitors of FIH can have the formula:

X [B]-[C]

where X is as defined above, B is a linker group and C is an entity binding to part of the CAD binding site of FIH, in general $CONH_2$.

B is typically a polymethylene group, generally having 6 to 8 carbon atoms or an equivalent group where one or more of the carbon atoms is replaced by a heteroatom, notably O, S or N and can be functionalised, for example with thiol, alcohol, carboxylate, hydroxamic acid or oxalate to mediate Fe binding. It is preferably 6 to 8 carbon atoms long or its equivalent. Alternatively, B is a linking group which possesses a ring, preferably of 5 to 7 members to which C is attached.

Inhibitors that bind to the 2OG binding site or part thereof and the peptide substrate

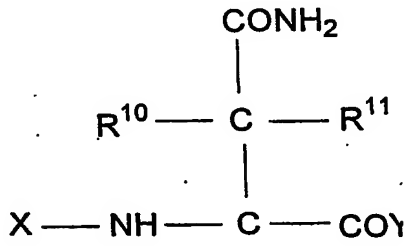
Another class of inhibitors bind to the enzyme-substrate complex, i.e. to FIH.Fe(II).HIF(CAD). The structural analyses enable the identification of such inhibitors. As described above 2OG and CAD binding sites are linked to each other via a 'channel' extending from the 2-oxo group of 2OG (or NOG) to the beta-carbon of Asn-803 in the FIH.Fe.2OG/NOG.HIF(CAD) complexes. Inhibitors of this type may be defined as X-[B]-[E] where X is as defined above, B is a linker group such as defined above and E is an entity binding to part of the CAD when bound to HIF. E may bind to the backbone carbonyl oxygen of Asn-803 of CAD and to the NH_2 group of the primary amide of Asn-803.

Mechanism based inhibitors

Another class of inhibitors is based upon substrate analogues that can undergo part of the catalytic cycle but either stall at an intermediate stage or cause an aberrant

reaction resulting in damage or inhibition. The observation that FIH catalyses hydroxylation of Asn-803 at the beta-position together with the structural analyses enables the design of such inhibitors. Such compounds include analogues of the substrates (inhibitors) in which Asn-803 is replaced with an analogue which does not undergo oxidation such as beta-fluoro- asparagine, beta-di-fluoro- asparagine, beta-methyl- asparagine, beta-dimethyl- asparagine derivatives. Alternatively derivatives that undergo oxidation to give an agent that can be oxidised to give an inactivating group such as an epoxide or metal chelating group may prepared (such mechanism based inhibitors are sometimes referred to as suicide inhibitors). In the case of FIH they include alpha-beta-dehydroasparagine and beta-methylene asparagine.

These include a compound having the formula



wherein X represents a valine residue or an analogue thereof and Y represents an alanine residue or an analogue thereof, R^{10} is fluorine or C_1 - C_3 alkyl, especially methyl, and R^{11} is fluorine, C_1 - C_3 alkyl or hydrogen i.e. the specified residue is β-mono- or di-fluoroasparagine or β-mono- or di-methylasparagine.

Alternatively, the compound above may be desaturated, i.e. is an alpha/beta dehydroamino acid (R^{11} not present) or R^{10} and R^{11} may be replaced by a methylene group, i.e. the residue is α, β-dehydro-asparagine or β-methylene asparagine.

If desired the valine residue is connected to one or more units of the peptide DESGLPQLTSYDCE - in the order given e.g. to glutamic acid (E) alone or to, for aspartic acid (D) - cysteine (C) - glutamic acid (E)-, or a longer chain such as PQLTSYDCE -.

For the compounds of this invention suitable aryl rings include phenyl and napthalenyl, which may be further functionalised or fused to other ring systems. Suitable heterocyclic rings include thiophene, pyridine, quinoline, isoquinoline, pyrimidine, pyrazine, pyrone, chromone, coumarin, indole, isoindole, indolizine, benzofuran, pyridazine, purine, oxazole, pyrazole, isothiazole, pyrrolidine, piperidine, indoline, benzothiaphen, morpholine, benzimidazole, azepine, azacine, azoine, oxepine, oxocine, oxoine, piperazine, oxazine, thiazine, thiepine, thiocine, thioine, furan, imidazole, azole, diazole, triazole and tetrazole ring systems that may be functionalised or fused to other ring systems.

The said alkyl and aryl groups and chains are typically functionalised by alcohol, fluorine, thiol, a carboxylic acid, phosphonic or phosphinic acid, sulphonic acid or other chelating group, in the case of the chains typically via an alkyl group. In the formulae described herein, a branched or straight C_1 to C_6 alkyl chain may be a methyl, ethyl, propyl, butyl, iso-butyl, *tert*-butyl, pentyl, neopentyl, *tert*-pentyl or a primary, secondary or tertiary hexyl group. Preferably the alkyl groups are methyl, the preferred heterocyclic rings are pyrolidine and tetrahydropyran and the preferred aromatic rings are benzene, naphthalene and pyridine.

The compounds which are acids can be present in the form of salts, such as sodium salts.

Therapeutic Applications

For therapeutic treatment, the compound may be used in combination with any other active substance, e.g. for anti-tumour therapy another anti-tumour compound or therapy, such as radiotherapy or chemotherapy.

Generally, the modulator is provided in an isolated and/or purified form, i.e. substantially pure. This may include being in a composition where it represents at least about 90% active ingredient, more preferably at least about 95%, more preferably at least about 98%. Any such composition may, however, include inert carrier materials or other pharmaceutically and physiologically acceptable excipients, such as those required for correct delivery, release and/or stabilisation of the active agent. Typically the concentration of modulator in such compositions is 0.1 to 50%, generally 0.5 to 20%, especially 1 to 10%, by weight based on the weight of the composition. As noted below, a composition according to the present invention may include in addition to an modulator compound as disclosed, one or more other molecules of therapeutic use, such as an anti-tumour agent.

In general they take the form of compositions wherein the compound is in a mixture with a pharmaceutically acceptable carrier or diluent. The carrier may be liquid, e.g. saline, ethanol, glycerol and mixtures thereof, or solid, e.g. in the form of a tablet, or in a semi-solid form such as a gel formulated as a depot formulation or in a transdermally administrable vehicle, such as a transdermal patch.

The invention further provides a method of treatment which includes administering to a patient compound as defined above. Exemplary purposes of such treatment are discussed elsewhere herein.

The therapeutic/prophylactic purpose of such a method or use may be the modulation of the level of HIFα in a cell by modulation, e.g. disruption or interference, of the hydroxylation of HIFα. Hydroxylation of HIFα promotes pVHL binding which leads to ubiquitin dependent proteolysis of HIFα as described above.

The therapeutic/prophylactic purpose may be related to the treatment of a condition associated with reduced or suboptimal or increased HIF levels or activity, or conditions where an alteration in HIF levels or activity may be beneficial such as:

(i) ischaemic conditions, for example organ ischaemia, including coronary, cerebrovascular and peripheral vascular insufficiency. The therapy may be applied in two ways; following declared tissue damage, e.g. myocardial infarction (in order to limit tissue damage), or prophylactically to prevent ischaemia, e.g. promotion of coronary collaterals in the treatment of angina.

(ii) wound healing and organ regeneration

(iii) auto-, allo-, and xeno- transplantation.

(iv) systemic blood pressure

(v) cancer; HIFα is commonly up-regulated in tumour cells and has major effects on tumour growth and angiogenesis.

(vi) inflammatory disorders.

(vii) pulmonary arterial blood pressure, neurodegenerative disease.

Pharmaceutical Compositions

In various further aspects, the present invention thus provides a pharmaceutical composition, medicament, drug or other composition for such a purpose, the composition comprising one or more compounds of formulae (A) to (F), or derivatives thereof, the use of such an composition in a method of medical treatment, a method comprising administration of such a composition to a patient, e.g. for treatment (which may include preventative treatment) of a medical condition as described above, use of such an agent compound or substance in the manufacture of a composition, medicament or drug for administration for any such purpose, e.g. for treatment of a condition as described herein, and a method of making a pharmaceutical composition comprising admixing such an agent, compound or substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

The agent may be used as sole active agent or in combination with one another or with any other active substance, e.g. for anti-tumour therapy another anti-tumour compound or therapy, such as radiotherapy or chemotherapy.

Whatever the agent used in a method of medical treatment of the present

invention, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

An agent or composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated, e.g. as described above.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous. The compositions will typically be sterile.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability.

Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants

and/or other additives may be included, as required.

Liposomes, particularly cationic liposomes, may be used in carrier formulations. Examples of techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

The substance or composition may be administered in a localised manner to a particular site or may be delivered in a manner in which it targets particular cells or tissues, for example using intra-arterial stent based delivery.

Targeting therapies may be used to deliver the active substance more specifically to certain types of cell, by the use of targeting systems such as antibody or cell specific ligands. Targeting may be desirable for a variety of reasons, for example if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

The compounds used as inhibitors are known or novel and can be made using known processes or processes which are analogous to known processes.

In a further embodiment the invention provides for the use of an agent of the invention in the manufacture of a medicament for the treatment of a condition associated with increased or decreased HIF levels or activity. The condition may, for example, be selected from the group consisting of ischaemia, wound healing, auto-. allo-, and xeno- transplantation, systemic high blood pressure, cancer, and inflammatory disorders.

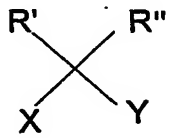
CLAIMS

1. A compound of one of the following groups for use in a method of treatment of the human or animal body by therapy:

(i) a thiol, alcohol, phenol, carboxylate, hydroxamate, imidazole or other heterocyclic compound, that binds to iron;

(ii) R-entiomer of N-oxaloylalanine, procollagen prolyl-hydroxylase and a PHD isozyme;

(iii) a compound of the formula

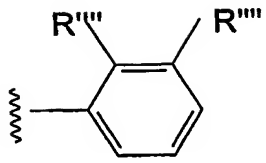


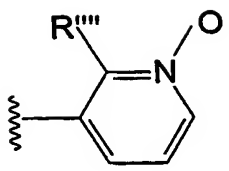
(I)

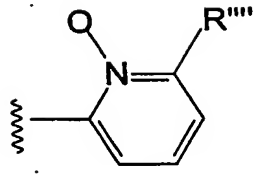
wherein each of R' and R", which may be the same or different, is H, F or C_1 to C_3 alkyl or substituted alkyl, CH_2OH, CH_2CO_2H or $CONH_2$, X is COOH, SOOH, or CONHH or an ester thereof, or other group which forms a favourable interaction with one or more of the side chains of Lys-214, Thr-196 and Tyr-145,

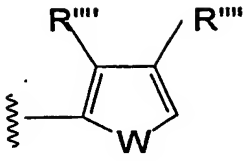
Y is - $(CR'''R''')_nZ$, where Z is

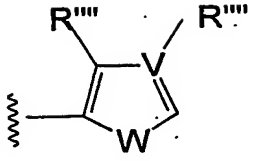
- $NR'''COCOOH$, $-NR'''CSCOOH$, - $NR'''COCOSH$, - $CHSR'''CONR'''R''''$, $-CHOR'''CONR'''OR'''$, - $CHSR'''CONR'''OR'''$ or - $CHOR'''CONR'''NR'''OR'''$, wherein each R''', which may be the same or different, is H, alkyl, OH or O-alkyl, n is 0 to 3, or





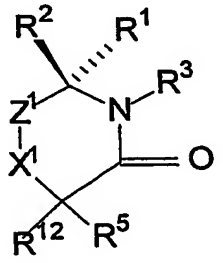






wherein R'''' is OH, OR''' or NHCOR''', and W is S, NH, or O;

(iv) a compound of the formula

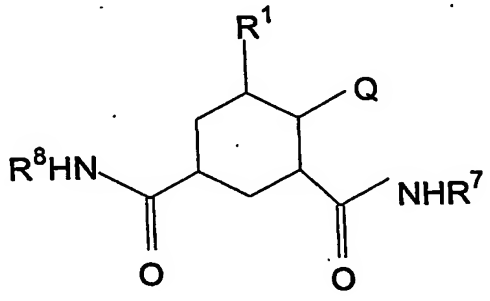


II

5

wherein R^1 is such that it can form an electrostatic or H-bonding interaction with Gln-237 and or Arg-238 R^2 is H or a group which reacts favourably with Tyr-102 of FIH, R^3 is H or a group which can form a H-bond with Asp-201, R^5 is hydrogen, optionally functionalised alkyl, aryl or heteroaryl or any combination thereof, R^{12} is as defined for R^5 or is NHR^6 where R^6 is COR^5 or SO_2R^5, and X^1 is NR^3, $NR^4C(R^5)_2$, $C(R^5)_2NR^4$, or O or NH where R^4 is COR^5 or $-SO_2R^5$ and Z^1 is $\rangle C=0$ or $\rangle CR^5R^9$ where R^9 is hydrogen, optionally functionalised alkyl, aryl or heteroaryl or any combination thereof;

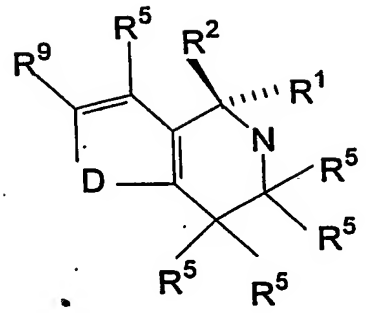
(v) a compound of the formula



III

where Q represents H or OH and R^7 and R^8 are independently peptides or peptide mimetics;

(vi) a compound of the formula



IV

wherein R^1, R^2, R^5 and R^9 are as defined above and D is S, O, NH or $CHR^9=CHR^5$;

(vii) a compound of the formula

X[B]-[C]

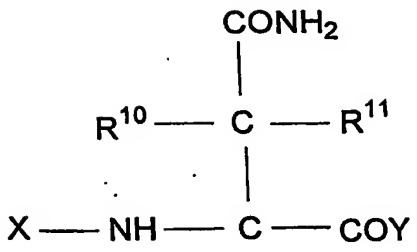
where X is as defined above, B is a linker group and C is an entity binding to part of the CAD binding site of FIH;

(viii) a compound of the formula;

X-[B]-[E]

where X and B are as defined above and E is an entity binding to part of the CAD when bonded to HIF; and

(ix) a compound of the formula



wherein X represents a valine residue or an analogue thereof and Y represents an alanine residue or an analogue thereof, R^{10} is fluorine or C_1 - C_3 alkyl, and R^{11} is fluorine, C_1 - C_3 alkyl or hydrogen or a corresponding compound R^{11} is absent or R^{10} and R^{11} form a methylene group.

2. A compound according to claim 1 for use in the treatment of a condition associated with increased or decreased HIF levels or activity or the treatment of a condition where it is desired to modulate HIF activity.

3. A compound according to claim 2 wherein said condition is ischaemia, wound healing, auto-, allo- or xeno-transplantation, systemic high blood pressure, cancer or an inflammatory disorder.